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A PLATELET SUBSTITUTE - THE PLATELETSOME TO BE USED IN TRANSFUSION THERAPY

FINAL REPORT

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This project was defined as a feasibility study to establish a foundation on which a liposome based platelet substitute could be developed. In the first year of this contract, platelet membrane glycoproteins (GP) and IIb-IIIa were isolated and incorporated into phospholipid vesicles as a heterodimer complex. GP Ib was purified by Triton solubilization of platelet membranes and affinity chromatography. The outside-out orientation of these glycoproteins in the liposomal vesicles, which were prepared by reverse-phase sonication, was confirmed by antibody and ligand binding studies. The calcium channel function of GPII-IIIa in liposomes and ligand binding inhibition of this channel were characterized with Fura-2 loaded liposomes. The ability of these GP bearing liposomes to form aggregates and to participate in platelet aggregate formation was confirmed. The expression of neoantigens on the surface of these liposomes was assessed. No antibodies to neoantigens could be detected by the immunolysis assay in rabbits immunized with GPIIb-IIIa liposomes or solubilized GPIIb-IIIa. GPIIb-IIIa liposomes did not activate humoral coagulation in vitro or in vivo

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ABSTRACT (continued): and did not induce platelet aggregation in vitro. A model system was developed to evaluate the adhesive properties of glycoproteins bearing liposomes in vitro under gravity and shear conditions. It was determined that maximum adhesion occurred with a composite liposome preparation bearing multiple platelet membrane GP. The adhesion of liposome preparations was determined by binding of radiolabeled liposomes. Adhesion of GPIIb-IIIa liposomes required surface fibrinogen and von Willebrand factor. In vivo models for the evaluation of the hemostatic efficacy of liposomes were developed. These models included the rat tail bleeding time in thrombocytopenic and thrombocytopathic rats. Thrombocytopenia was induced by external Cesium irradiation, 200-900 rads. The thrombocytopathic rat was the Fawn-Hooded rat, an in-bred strain with congenital thrombocytopathy (platelet storage disease). Bleeding times and blood loss in these rats was significantly greater (>20 times) control rats. We determined that a liposome preparation based on a composite preparation of 12 different platelet membrane GP caused significant amelioration of bleeding in both animal models (42% reduction). These composite liposomes did not induce thrombosis in these animals nor in normal animals into which they were infused as documented by post mortem examination with microscopy of this. These studies document the feasibility of this general approach to a platelet substitute and support continued investigation.

FOREWORD

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INTRODUCTION

Platelets have a central role in hemostasis and have been implicated in pathologic processes such as atherosclerosis, myocardial infarction, stroke and tumor metastases (1-4). Platelet transfusions are essential in the treatment of severe trauma which is complicated by significant blood loss and massive transfusion with red blood cells and colloid and in the treatment of other causes of thrombocytopenia. Platelet transfusions are associated with limitations which include: relative shortage, secondary to a dearth of donors and the relatively short in vitro survival of platelet concentrates; loss of platelet viability and functional capability during storage; and risk of transmission of infections such as non-A non-B hepatitis, hepatits B and human immunodeficiency viruses. In addition, alloimmunization, which occurs in 10-30% of transfusion recipients, is a significant problem.

Major advances have been made in the understanding of platelet physiology. Platelet membrane GPIIb and IIIa have been characterized as a Ca^{2+} dependent heterodimer complex (5-9) that belongs to a family of adhesive protein receptors, the integrins (10,11). During platelet activation, this complex undergoes a conformational change and functions as a FGN receptor (12-19). This FGN binding is essential for platelet aggregation as demonstrated by the bleeding diathesis and absent platelet aggregation in Glanzman's thrombasthenia, an inherited deficiency of these GP (20-23), and by the ability of a number of MoAbs to this complex to inhibit platelet aggregation (24,25). GPIIb and IIIa have been cloned (26-30), amino acid (AA) sequences determined, homology to the vitronectin receptor defined,and some functional regions identified e.g. putative Ca^{2+} binding regions with structural homology to calmodulin (27-30). CHO chains of these GP have been determined (31) and a thrombasthenic variant with abnormal glycosylation has been described (32). FGN binding properties of the soluble complex have been defined and purified complex inserted into liposomes (36,37, mid-term report) with preservation of FGN binding. The proposed sites on the FGN molecule that interact with the GPIIb-IIIa complex are a dodecapeptide (His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val: His-12-Val), residues 400-411 of the COOH terminus of the FGN gamma chain (38-40), and a tetrapeptide (Arg-Gly-Asp-Ser:RGDS) near the COOH terminus of the alpha chain or a tripeptide (Arg-Gly-Asp:RGD) near the NH_2 terminus of the alpha chain (41-46). The synthetic peptide analogues of these regions inhibit platelet aggregation and FGN binding to activated platelets and are mutually inhibitory in binding studies (41-47). The data suggest that these peptides share common or closely related binding sites on GPIIb-IIIa. RGDS is also a proposed sequence by which vWF binds to GPIIb-IIIa and to GPIb.

Ligand binding to GPIIb-IIIa may signal secondary events in the platelet and alter the conformation and distribution of the GPIIb-IIIa complex. Isenberg et al have reported that binding of FGN, RGDS, or the gamma dodecapeptide induces clustering of platelet GPIIb-IIIa (48). Banga et al have reported that occupancy of GPIIb-

IIIa by FGN is necessary to maintain Na^+/H^+ exchange in epinephrine stimulated platelets (49). Parise et al have demonstrated that binding of LGGAKQAGDV or RGDS to soluble GPIIb/IIIa alters the hydrodynamic properties of the complex and renders GPIIb susceptible to thrombin hydrolysis (50).

Platelet membrane GPIb has also been extensively characterized. This glycoprotein contains a receptor for vWF (51). Ab directed against GPIb prevent vWF binding to the platelet surface and inhibit vWF-dependent ristocetin induced platelet agglutination (52). This GP exists in the membrane in complex with GPIX; this complex can remain associated following detergent solubilization of the platelet membrane (53). In addition, GPIb has been reported to be the receptor for quinine/quinidine drug dependent antibodies (54) and to bind thrombin. Thrombin and vWF receptor function may partially reside on the glycocalicin (GC) portion of GPIb, a protease sensitive region (55). Berndt (56) and Canfield (57) have reported methods for purifying GPIb and GPIb-IX free of contaminating proteins. These purified proteins have been bound to solid substrate (58) and partially purified proteins incorporated into liposomes (59). The amino acid sequence of GPIb has been obtained by cDNA (60,61). GPIb is heavily glycosylated but the role of carbohydrate in functional properties is not defined.

In addition to platelet GP, platelet cytoplasmic ionized calcium concentration, $[Ca^{2+}]_i$, plays a central role in platelet activation. Several platelet agonists, such as thrombin, ADP, and platelet activating factor produce an increase in platelet $[Ca^{2+}]_i$ (62-66) which occurs on a subsecond time scale. Most of this Ca^{2+} is derived from intracellular sites such as the dense tubular system, although activation is also associated with increased platelet plasma membrane permeability to Ca^{2+} (61-68). The role of extracellular calcium in human platelet aggregation has not been precisely defined. However, even in unstimulated platelets in which the Ca^{2+} concentration is 10,000 fold less than plasma, Ca^{2+} exchange can occur across the plasma membrane. The potential role of the GPIIb-IIIa complex in platelet Ca^{2+} flux is supported by data that the maximum rate of platelet plasma membrane $^{45}Ca^{2+}$ exchange in thrombasthenic platelets is half that observed in normal platelets (69,70) and that dissocia- tion of the GPIIb-IIIa complex in normal platelets causes a decrease in basal $^{45}Ca^{2+}$ influx. Studies with MoAb and a pentapeptide ligand by Yamaguchi (71) and Sinigaglia (72) and our studies with GPIIb-IIIa incorporated into liposomes (35,73 midterm report) provide additional evidence that this GP complex may play a role in platelet Ca^{2+} flux. The role of CHO in this channel, and for the heavily glycosylated channels in other cells (74), is undefined. Jennings has described abnormalities of Ca2+ mobilization in thrombasthenic platelets (75); however, from MoAb studies with intact platelets, Powling et al postulated that a platelet plasma membrane calcium channel is located in proximity to the GPIIb-IIIa complex but that it is not GPIIb-IIIa (76,77).

A number of critical questions remain. These questions include the role of transmembrane influx of external Ca^{2+} during platelet

activation, the contribution of GPIIb-IIIa to this influx, regions of GPIIb/IIIa involved in channel function, the potential for ligand binding to terminate Ca^{2+} influx, and the role of CHO in ligand binding and the Ca^{2+} channel function of the GPIIb-IIIa complex. In addition, platelet GPIb is essential for platelet adhesion; however, the role of CHO and lipid milieu on function are not defined. The contribution of GPIIb-IIIa to adhesion, particularly under shear conditions, and the impact of lipid environment on adhesion require clarification. Characteristics of platelet GP in artificial membranes require further elucidation. Enhanced understanding of platelet physiology will facilitate development of platelet inhibitors for clinical use. Furthermore, the current model may be applicable to other members of the integrin family of proteins. Development of models of the platelet will also contribute to the development of a platelet substitute to be used in transfusion therapy. The latter assumes greater importance daily due to increasing use of platelet transfusions, the relative shortage of platelet concentrates and the risk of disease transmission by such concentrates. The current contract was designed as a feasibility study to evaluate a liposome-based platelet substitute. A general outlin e of our original proposal is shown in Table 1.

MATERIALS AND METHODS

I. Glycoprotein preparation

A. GPIIb-IIIa Preparation:

Glycoproteins IIb-IIIa were prepared by modification of published methods (37,78,79) from Triton X-114 solubilized platelet membranes. Platelet concentrates in acid-citrate-dextrose anticoagulant were washed x 2 in citric acid buffer (0.013M citric acid, 0.013M sodium citrate, 0.033M D-Glucose) and NaCl (150mM), pH 7.0 and washed x 2 in Tris buffer (0.01M Tris, and 0.15M NaCl, pH 7.24). Washed platelets were resuspended in Tris buffer (0.01M) pH 7.4 with $CaCl_2$ (0.5mM) and NaCl (0.15M) with phenylmethylsulfonyl-fluoride (PMSF) (0.4mM) and leupeptin (100ug/ml). Platelets were sonicated on ice with a probe sonicator and centrifuged (1000xg) to remove non-disrupted platelets. The supernatant was centrifuged at 78,000 xg for 1 hr and the pellet from this centrifugation suspended 1% (V/V) in precondensed Triton X-114, 10mM Tris, NaCl (150mM) buffer pH 7.4 with 0.4mM PMSF, incubated overnight at 4°, and centrifuged at 78,000xg for 1 hr at 4°C. The detergent phase was then applied to a 6% sucrose cushion, heated at 37° for 5 minutes, centrifuged at 1500xg for 5 minutes, and the detergent micelle aggregate layer removed. Some variability of protein composition of Triton X-114 preparations was observed. Further purification of proteins was achieved by application of the detergent phase to lentil-lectin Sepharose, and elution with 10% alpha-methyl-D-mannoside in Tris HCl buffer, NaCl 150mM, 10^{-6}M $CaCl_2$ pH7.4. Detergent was partially removed with Biobeads SM2. Each GPIIb-IIIa preparation was assayed for total protein by modified Lowry (80) and residual Triton X-114 determined spectrophotometrically (A_{622}) after reaction with ammonium cobaltothiocyanate reagent and solubilization in ethylene chloride (81). Preparations maximally contained 0.27-0.90 mg Triton per mg protein. The proteins were then incubated in 10^{-6}M Ca^{2+} at 25°C for 1 hour prior to incorporation into vesicles to ensure consistent, albeit incomplete, association of the complex without excessive Ca^{2+} loading of vesicles. For dissociated complex studies, the proteins were incubated in Ca^{2+} free buffer and initally prepared with EDTA (0.001M) in platelet membrane washing buffers, then maintained in Ca^{2+} free buffer. This resulted in GPIIb which was 97% cleavable by thrombin, consistent with complex dissociation (50).

Proteins were analyzed by SDS-PAGE, reduced and non-reduced, using 7.5% gels. While small amounts of contaminating proteins theoretically may have been present, they were not detectable by combined silver and Comassie blue stains of gels of any GPIIb-IIIa preparation used. This was presented in the mid-term report.

B. Composite proteins

Platelet membranes were prepared as described above. These membranes were then solubilized with 0.5% NaDeoxycholate (DOC). Detergent was removed by extensive dialysis at 4°C. SDS-PAGE of this

protein preparation included 12 bands which included bands consistent with GPIb,IIb,IIIa and Ia.

II. Proteoliposome Preparation

Large unilammelar vesicles were formed from egg lecithin (phosphatidylcholine) (Sigma Chemical Co., St. Louis, MO) by reverse-phase method (38,82,83, mid-term report). Phosphatidylcholine vesicles permitted use of varying Ca^{2+} concentrations without vesicle fusion, or microscopic aggregate formation, and limited intrinsic cation permeability. Phosphatidylcholine (25mg in hexane at 100mg/ml) was dried to a thin film on the bottom of a 50ml round bottom flask in vacuo, 3-14 hours. The lipid film was resuspended in 1.5ml of ether and then 0.250ml of 10mM tris HCL or 10mM Hepes, 150mM NaCl buffer, pH7.4, plus 30uM Fura-2 penta potassium salt (Molecular Probes Inc, Eugene, OR) for influx studies. This mixture was then sonicated for 2 min in a bath type sonicator, containing ddH_2O with 0.01% Triton. Sonication was carried out at the point where the surface of the bath exhibited maximum agitation. The ether was removed by rotary evaporation under reduced pressure to form a lipidic gel. This gel was sonicated for 1 min with 1 ml of buffer containing glycoproteins (50-700ug/ml) + Fura 2 which had been kept at 4°. Liposomes were washed x 2 by centrifugation at 14,000 xg for 10 min at 4°C.

These liposomes were relatively homogenous in size and entrapped volume with mean diameter of 5 microns as determined by laser light scattering with a Coulter N4 Submicron Particle Sizer.

For proteoliposomes of smaller size, required for in vivo studies, an additional step of French press extruison at 20,000 psi was added. Thses lipsomes were 200-900A in diameter.

Outside-out orientation was determined by vibrio cholera neuraminidase cleavage of sialic acid residues (36). SDS-PAGE of proteins associated with SDS-solubilized liposome preparations, revealed only 2 bands consistent with IIb and IIIa and 12 bands with composite lipsomes. Studies of Ca^{2+} influx which involved the dissociated complex were performed at equivalent protein/lipid ratio per liposome as the reassociated complex.

To confirm that Fura-2 was retained in the liposomes following centrifugation, liposomes were gel filtered on a 6ml Sephadex G-50 column. Fura-2 fluorescence (ex 345nm; em 510nm) was measured. An equivalent amount of Fura-2 remained within control and GPIIb-IIIa complex liposomes. This indicates that Fura-2 does not leak preferentially from the protein liposomes during the course of the experiments.

Liposome stability was determined with entrapped carboxyfluorescein (CF). Liposomes were prepared with 100mM entrapped CF; entrapped CF was separated from free CF by filtration of liposomes on a Sephadex G-50 column. Liposomes were then incubated in plasma or Hepes buffer pH 7.40 with 10mM Ca and 150mM NaCl. Aliquots were

removed, and liposome associated CF fluorescence measured (ex 410nm, em 520nm) before (F_t) and after (F_T) release of entrapped CF with liposomes Na deoxycholate (0.5% final). The fraction of CF remaining in the vesicles has been shown previously to equal 1-Ft/FT.

III. Calcium Transit Studies

In all cases liposomes were utilized the same day of preparation and kept on ice until use. For Fura-2 studies, liposomes were used within 1 hour of preparation. Temperature and pH were rigorously controlled in all experiments and double-distilled deionized 18 milliohm water utilized in all buffers.

For Ca^{2+} movement into vesicles, Fura 2 (30uM) loaded liposomes (10^{-6}M intravesicular Ca^{2+}) were injected into buffer (20ul of liposomes suspension to 980ul of Tris(10mM), NaCl(150mM) buffer $\pm$ Ca^{2+} (10^{-5}-10^{-2}M); this approximates infinite-cis-entry conditions (saturating external calcium). The Fura-2 fluorescence was measured (ex 345nm, em 510nm) in a Perkin-Elmer 650-10S Fluorescence Spectrophotometer with a Perkin-Elmer 150 Xenon power supply. These emission and excitation wavelengths minimized contribution from intrinsic liposomal fluorescence and beam dispersion by the liposomes. These factors precluded an accurate determination of Fmax and Fmin for Fura-2 in this system. A standard curve of fluorescence vs intravesicular (Ca^{2+}) was established using non-protein liposomes which were loaded with 30uM Fura-2, known calcium concentrations (10^{-6} to 10^{-2}M) and fluorescence measured. This is shown in figure 2. Effect of extravesicular NaCl concentration was determined by measuring Ca^{2+} influx as described above in buffers containing 10-150mM NaCl. Intravesicular NaCl was maintained at 150mM or osmolarity maintained with N-methyl-D glucamine. To assess ionomycin effects on Ca^{2+} entrance into vesicles, 1uM ionomycin was added to liposome suspensions in Ca^{2+} containing buffer followed by measurement of fluorescence.

IV. Adhesion

Adhesion of ^{3}H-PC liposomes (control, GPIIb-IIIa, Composite) to deendothelialized bovine and human aorta, and to microtiter wells. Briefly, a 200ul suspension of ^{3}H-liposomes (100-200x10^3 cpm, 12ug lipid) was added to bovine or human aortic segments, which had been gently scraped to ensure deendothelialization, or to microtiter wells. The surface to be tested was preincubated with buffer, cryoprecipitate, collagen, FGN, FNC, or vWF. Liposomes were preincubated with buffer or plasma. Prior to plasma incubation, we showed that liposomes retained integrity (as determined by carboxyfluoroscein retention within liposomes) in plasma. Following incubation at 25°C, segments or wells were gently washed and adhesion quantitated by measurement of residual counts. The amount of bound lipid was then calculated. GPIIb-IIIa liposomes and composite liposomes were evaluated for

a. substrate specificity (collagen, deendothelialized aorta, cryoprcipitate vWF, FGN)

b. resistance to shear stress

1) Proteoliposomes were prepared by reverse-phase/sonication and for smaller liposomes (200-900A), an additional step of high pressure extrusion by French Press was added. An alternative approach for smaller liposomes is extensive sonication; however, this may denature the incorporated protein. Radiolabelled liposomes were prepared with ^{3}H-PC.

2) Adhesion under shear stress was measured with a polymethacrylate chamber modified from Sakariassen et al (84). The flow in this chamber enters in a cylindrical tube with the inlet oriented at 20° to a rectangular cross section with a depth of 0.195± 0.005mm and width 18mm (to contain coverslips 18x18x0.147± 0.003mm or thin tissue sections) with a removable central knob. The inlet is connected to an injection syringe for controlled-rate injection. Coverslips were coated with delipidized BSA, purified protein (Collagen, vWF, FGN, FNC) plus BSA (to block residual sites) or thin tissue sections of deendothelized human (post-mortem) and bovine aorta. Liposome suspensions were infused through this system, adherent liposome-^{3}H counted in a scintillation counter and total adherent lipid was quantitated.

3) Non-shear adhesion was by the method of Pytella et al (10). ^{3}H-liposome suspensions were allowed to settle (3 hours) on microtiter wells (Linbro 96 flat bottom wells 1.0x0.6 cm, Flow Laboratories Inc) precoated with BSA or purified protein (Collagen, vWF, FGN, FNC) plus BSA (to block residual sites), or containing circlets (10-50mm diameter) of deendothelialized bovine or human aorta (histologically confirmed) with and without FGN and vWF. After incubation, the wells were gently washed, fluor added, and ^{3}H counted. All experiments were performed with at least 10 replicates and repeated at least 3 times in order to attain statistical significance.

V. Immunogenicity

Female (6lb) New Zealand White rabbits were immunized (2 animals per immunogen) with: phosphatidylcholine liposomes (12.5mg lipid), IIb-IIIa liposomes (100ug protein in 12.5mg lipid), solubilized GPIIb-IIIa (100ug), or activated platelets. All antigens were emulsified in complete Freund's adjuvant (Liposomal integrity was preserved). Prior to immunization, rabbits were observed for 1 week and preimmunization sera were obtained from an arterial puncture to determine presence of natural antibodies. The immunization procedure involved one injection into the popliteal lymph node, exposed by surgical dissection under anesthesia. For this procedure, animals were anesthetized with I.M. Ketamine (35mg/kg) and Rompun (5mg/kg). Evans blue dye was injected between the toes to facilitate visualization of the lymph node. this method has been shown to enhance immune response to small amounts of antigen. Additional rabbits received primary immunizations with intravenous injections of soluble IIb-IIIa or intact human platelets (100,000/ul). Three subcutaneous boosts were given at 3 week intervals with antigens emulsified in incomplete Freund's adjuvant. Rabbits, which had received intravenous primary immunization,

received intravenous boosts. Rabbits were bled every 7-10 days. Sera were absorbed with bromolinated human red blood cells to remove anti-human antibodies and then tested by immune lysis assay.

VI. Immune Lysis Assay utilized a modification of the glucose release method of Kinsky (85,86). Before testing, antisera were heated at 56°C for 30 min. to inactivate complement and then were centrifuged at 27,000g for 1hr to remove aggregates and low density lipid. Antisera were absorbed with bromolinated human RBCs to remove anti-human antibodies, and, in selected experiments, with human platelets. Fresh human sera were used as a source of complement. All sera were extensively dialyzed to remove glucose. Sera were frozen at -70°C in aliquots until testing.

Glucose release assay reagent: both complete assay reagent (CAR) and incomplete assay reagent (IAR) were prepared in Tris buffer pH7.5 with NaCl, $MgCl_2$, $CaCl_2$ with hexokinase(30ug/ml Boehringer-Mannheim, dialyzed to remove ammonium sulfate), glucose-6-phosphate dehydrogenase (15ug/ml, dialyzed), ATP (92mM), NADP(TPN)(1mM) for CAR. IAR did not include NADP, therefore it is used as a blank. The assay reagent was freshly prepared daily.

Liposomes were prepared as described above but with 0.3M entrapped glucose. Prior to use, liposomes were washed to remove untrapped glucose. The amount of residual untrapped glucose was measured as described below.

The final assay mixture included: 0.5ml glucose assay reagent(CAR or ICAR), 0.355ml of 0.15MNaCl, 0.2ml of antiserum to be tested, 0.12ml complement and 0.005ml of liposomes (control or IIb-IIIa).

Two A340 readings were recorded: pre and 30 min post addition of liposmes.

To measure total glucose, duplicate test tubes were set up containing 0.005ml of liposomes and 0.5ml of chloroform (to release entrapped glucose;removed by a stream of N_2) and either CAR or ICAR and buffer added. The $A340_{CAR}$ minus $A340_{ICAR}$ (background) equals total glucose. Untrapped glucose was measured in a similar manner without the addition of chloroform, with correction for liposome absorbance.

The glucose released by the test serum is expressed as per cent trapped glucose released :

$$= \frac{\text{A340 final assay mixture(CAR)-background}}{\text{A340 trapped glucose}}$$

$$= \frac{\text{A340 final assay mixture(CAR)-A340 final mix(ICAR)}}{\text{A340 chloroform releasable-A340 untrapped}}$$

VII. Patient PILA studies

Serum samples were obtained from the clinical pathology laboratory or blood bank from patients with immune mediated thrombocytopenia, patients with alloimmunization to platelet transfusions, patients with HIV associated thrmbocytopenia, and patients wit non-immune thrombocytopenia (e.g. bone marrow failure, chemotherapy, disseminated intravscular coagulation). Sera were assayed in a blind manner in the PILA as described above with and

without prior absorbtion with non-protein PC liposomes.

VIII. Effect on coagulation in vitro and in vivo

Due to concern over the possibility of massive activation of platelets or of humoral coagulation in vivo by liposomes bearing platelet membrane proteins, proteoliposomes were assessed in standard in vitro assays: prothrombin time (PT) and partial thromboplastin time (PTT) using automated techniques (Coagumate) with kaolin and thromboplastin reagent as activators; the whole blood clotting time was maesured by mechanical resistance. Platelet aggregation (spontaneous, collagen 0.8-16ug/ml, ADP 1.6-32uM, epinephrine 5-40uM and arachidonic acid 200-500uM induced) was measured with a dual channel aggregometer. Blood was anticoagulated with Na citrate (0.011M) and platelet rich plasma (PRP) prepared by centrifugation (15 Min, 100xg). Spontaneous and agonist induced aggregation with and without liposomes was measured. Initial rate of aggregation and degree of responsiveness were measured.

To study in vivo effects, fibrinogen concentration and platelet counts were obtained in rabbits following intravenous administration of control and specific liposomes.

Postmortem examinations were performed on thrombocytopenic and normal rats which had received liposome infusions.

IX. Effect of proteoliposomes on hemostasis in vivo in thrombocytopenic and thrombocytopathic, fawn-hooded (F-H), rat models

The hemostatic efficacy of proteoliposomes was assessed by infusion into the abdominal aorta of thrombocytopenic, F-H, and hematologically normal rats. Effect on hemostasis was quantitated by the rat tail bleeding time.

1. Thrombocytopenic rat model Thrombocytopenia was induced in male outbred Sprague-Dawley rats (200-400g weight necessary to facilitate catheter insertion into the aorta) by external TBI with a Cesium source. Rats were irradiated with 0-900 rads and serial platelet counts were determined. Platelet counts were measured on days 0-14 by amputation of a 0.5cm segment from the tail and collection of 50ul of blood in EDTA (0.2% final), and platelets counted with a Baker Series 810 Platelet Analyzer adjusted to rat platelet volumes (1.7-18u^3). Repeated dose respose curves were performed to determine the minimum dose required to induce thrombocytopenia. A limitation of this approach is potential vascular endothelial cell damage, gastrointestinal toxicity, the development of anemia. To avoid the former problems, minimal radiation doses were utilized. Transfusion with rat RBC could correct the anemia; however, this was unnecessary as all data could be corrected for hemoglobin (Hgb) concentration. An alternative approach to induce thrombocytopenia is isovolumetric exchange transfusion with flourocarbon Hgb substitute. However, relatively short survival of animals with this approach makes it less desirable.

2. Thrombocytopathic (storage pool deficiency) rats were obtained from the New York State Department of Health. An asset of this model is that it mimics a different clinical situation; the animals, despite prolonged bleeding times, have platelets which theoretically can be activated. In addition, they may also have a minor disorder of humoral coagulation.

3. Assessment of hemostasis:rat tail bleeding time (RT-BT) In anesthetized (Ketamine, Rompun) rats, the femoral vein and artery were exposed. Catheters (22 gauge,Teflon) were inserted under dirct visualization. Hgb and platelet count were obtained venous sampling in each animal. Liposome suspension (control or protein) human platelets, plasma, or buffer were infused into the abdominal aorta of rats (minimum of 6 animals per group for statistical analysis). A RT-BT was performed, modified from Dejana (87) and Pertrasek (88). The tail was immersed in 0.15M NaCl, 37°C, after 3 min, a standardized incision (9mmx1mm) was made 5mm from the tip and the tail immersed for 30sec in 25ml of isotonic saline (37°C). The tail was then transferred to a series of tubes of 0.15M NaCl at 30 sec intervals. The bleeding time was measured from the moment the tail is cut until bleeding stopped completely. Initial, interval, and total blood loss, corrected for Hgb concentration, were performed spectrophotometrically after RBC lysis by the addition of Triton X 100 and freeze-thaw lysis. In addition to control groups, each animal served as its own control, i.e. a RT-BT was performed and sealed will Collodiun; 8 hrs later, the animal was infused and a BT incision made 180° from the first.

RESULTS

Protein orientation on the surface of the liposomes. GP IIb-IIIa liposomes, prepared by reverse-phase, bound monoclonal antibodies directed against GP IIb/IIIa (Table 2). On average, 70% of added protein is incorporated by either reverse-phase or freeze thaw preparation, with 48.6 ± 0.8% of protein in an outside-out orientation as determined by vibrio cholera neuraminidase susceptible sialic acid residues. The ability to insert these proteins in an out-side out orientation and for these proteins to remain as a hetero-dimer complex is an essential prerequisite to a potential platelet substitute. This has been achieved for GPIIb-IIIa.

GPIIb-IIIa on the surface of liposomes bound ^{125}I-fibrinogen with a Kd=10^{-7} and binding isotherm shown in figure 1. While this observation confirms the integrity of at least some of the complexes in the surface of the liposomes, it could prove to be problematic in localization of liposomes to a wound surface if these receptors are blocked with bound ligand.

GP IIb-IIIa were shown to mediate Ca^{2+} and fibrinogen dependent liposome aggregation. The characteristics of these aggregates are detailed in Table 3. Aggregation was inhibited by monoclonal antibody to the GPIIb-IIIa complex. Importantly for potential in vivo use, these aggregates did not occur spontaneously but required induction by centrifugation. Aggregate formation does confirm the possibility of inter-liposomal interactions to be mediated by glycoproteins. Aggregates were highly susceptible to disruption by shear forces which is important for safe in vivo use but suggests further modifications will be necessary for stable interactions to occur at a wound site.

Ca^{2+} transit studies When GPIIb-IIIa phosphatidylcholine liposomes with encapsulated 10^{-6} M Ca^{2+} and 30uM Fura-2, a fluorescent Ca^{2+} indicator, were incubated in buffer containing 10^{-5}-10^{-2} M Ca^{2+}, the GPIIb-IIIa complex but not dissociated GPIIb-IIa mediated Ca^{2+} transit across the phospholipid bilayer. These data are shown in Table 3. This Ca^{2+} transit was inhibitible by the synthetic peptide His-12-Val but not by RGDS (Table 4, fig 2); however, incubation of liposomes with RGDS prior to incubation with His-12-Val could preclude the His-12-Val inhibition (Table 4). This suggests that blockade of His-12-Val binding by RGDS prevents the inhibition. Similar experiments using ^{45}Ca influx in steady-state and ADP activated gel filtered platelets (GFP), showed a similar trend in inhibition; however, only a minor decrease in transmembrane Ca^{2+} influx was observed. Additionally, a His-12-Val dodecapeptide analogue HHLGGARQAGHDV which is relatively inactive in inhibition of platelet aggregation, failed to inhibit platelet ^{45}Ca influx.

Only 1 (M148) of a panel of MoAbs to GPII/IIIa tested caused significant channel inhibition despite documentation of binding. Hardisty has shown that M148 and F(ab')$_2$ but not Fab fragments of this Ab inhibits Ca influx into intact platelets (76,77). In

addition, this Ca^{2+} channel activity is blocked by addition of La^{3+} (10^{-3} M), a characteristic of other Ca^{2+} channels.

Liposome stability Stability of liposomes in buffer and in plasma were determined by use of encapsulated carboxyfluorescein. Due to the self-quenching property of this compound, release of intra-liposomal contents can be monitored over time. In addition, liposome size was montored; change is size is an index of rupture and fusion.

GPIIb-IIIa liposomes were found to be stable in buffer for 7 days and in plasma for 10-14 days. The results were identical for phosphatidylcholine and phosphatidylcholine:cholesterol (70:30: mole:mole) liposomes. These results are shown in Tables 5 and 6.

Activation of coagulation and of platelets GPIIb-IIIa liposomes had no significant effect on in vitro coagulation. These results are shown in Table 7. Proteoliposomes were able to participate in platelet aggregate formation, as determined by association of ^{3}H-GPIIb-IIIa liposomes but not control liposomes with activated platelets. Incorporation into platelet aggrgates wa 10-15% of liposomes prepared with 200ug of protein. No proteoliposome preparation tested, at 0-40% (v:v), which ranges from obtainable to above desirable in vivo concentrations, induced spontaneous platelet aggregation or changed the initial rate or final respnsiveness of platelets to agonists.

For in vivo assessment, animals were followed for the activation of coagulation after intravenous administration of GPIIb-IIIa and composite liposomes. There was no evidence of DIC, specifically platelet count and fibrinogen levels were unchanged (platelets/ul: pre:199,355; post: 167,445 for GPIIb-IIIa or composite liposomes; pre 195,024; post:116,388 for control; fibrinogen mg/dl-- pre 680$\pm$ 45, post 610$\pm$ 50 for GPIIb-IIIa or composite liposomes; no significant difference).

Adhesion

Experiments were performed to assess the adhesion of ^{3}H-PC liposomes (control, GPIIb-IIIa, composite) to de-endothelialized bovine and human aorta, and to microtiter wells coated with various substrates. Liposomes were preincubated with buffer or plasma. Prior to plasma incubation, we showed that liposomes retained integrity in plasma. GPIIb-IIIa liposomes adhered to cryoprecipitate treated aortic segments and to FGN but not collagen or BSA coated microtiter wells. These data are shown in Table 8. In these studies, cryoprecipitate, with presumed FGN/VWF binding, incubation was essential for GPIIb-IIIa liposome adhesion. Composite liposomes adhered with 1.5x the efficiency of GPIIb-IIIa liposomes. Incubation of lipsomes with plasma prior to adhesion studies blocked adhesion, possibly by FGN or vWF receptor blockade. Similar results were obtained with coated coverslips placed in a flow chamber. As anticipated, with shear stress, less (30% decrease) adhesion was observed. These data, which require further investigation do indicate that this is a useful approach by which to study the

adhesive properties of platelet GP.

Thrombocytopenic rat model. External total body irradiation (TBI) by external Cesium source was determined to be an effective modality with which to consitently induce thrombocytopenia in rats. Male, Sprague-Dawley out-bred rats (400g), after 2 weeks of observation, were given 0 (control), 200, 400, 600, 900 Rad TBI by Cesium unit. By day 10, the 900 Rad TBI group had a platelet count <20,000/ul and remained thrombocytopenic until day 14. Mean platelet counts are shown in Table 9. Less than 1% of animals died secondary due a single irradiation. However, 2/3 animals who received 2 treatments with 900 Rads, 7 days apart, died.

RT-BT Thrombocytopenia was associated with a prolongation of the BT. A reproducible method to determine the bleeding time in treated and control rats in which blood loss per min can be quantified and in which each animal can be used as its own control, as well as be compared to other groups of animals was developed. This is described in the Methods section. With this technique, normal animals had a bleeding time of 113 $\pm$ 3 sec with blood loss of 19ul in the first 30 sec, and 13ul in the next 6 min. Thrombocytopenic animals had a BT >6min, and blood loss at 30 sec: 102.9 $\pm$ 30ul, in the next 60 sec: 223.8 $\pm$ 58.5ul, and 369.7 $\pm$ 58.8ul in the next 6 min. (Table 10).

Fawn-Hooded rats have been characterized in this model. These rats have an apparent platelet storage pool deficiency as the major platelet abnormality. We found a BT >6min, blood loss at 30 sec: 143$\pm$20ul; next 60 sec: 212 $\pm$ 30ul, next 6 min, 458.3 $\pm$ 60ul. In these animals a minor increase in blood loss in the first 30 sec was noted following buffer infusion (Table 10).

Liposome infusion

The results of these investigations are presented in Table 10. Compared to control or buffer control, plateletsome infusion resulted in a significant decrease in blood loss in the first 30 sec, second 60 sec and total 6 min blood loss and also produced an increase time to half-maximal blood loss. Specifically, for thrombocytopenic rats, a 53.7% reduction in blood loss in the first 30 sec, a 67% reduction in the next 60 sec and a 43% reduction in 5 min blood loss was observed. In thrombocytopathic, Fawn-hooded, animals a similar 43% reduction in blood loss in the first 30 sec, a 31%reduction in the next 60 sec and a 50% reduction at 5 min was observed. No reduction in bleeding time was seen. Control (i.e. PC alone) lposomes had no effect on bleeding in either animal model.

These data indicate that these plateletsomes can effect hemostasis in vivo in this model although dose and preparation have not yet been optimized.

Post mortem examinations 1/2 hour after liposome injection revealed no evidence of thrombosis or pulmonary toxicity in normal or thrombocytopenic animals.

Immunogenicity

Expression of neoantigens when platelet glycoproteins are inserted into a new lipid environment is an important issue for the use of a liposome based moiety as a tranfusion modality. This has been addressed by immunization of rabbits with: liposomes, liposomes bearing GPIIb-IIIa on the surface, solubilized GPIIb-IIIa, and activated platelets. Antibody response was then compared by immune lysis assay as detailed in the Methods section.

Spontaneous anti-lipid antibodies were detected in rabbits which is characteristic of that species. Soluble GPIIb and IIIa, when administered intravenously and by popliteal lymph node injection, were highly immunogenic in rabbits as anticipated. Rabbits also developed increasing titers of anti-lipid antibodies which again is characteristic of that species. The time course of anti GP antibody development as determined by immune lysis assay is shown in figure 3. GPIIb-IIIa liposomes administered into the popliteal lymph node were more immunogenic than intravenously administered liposomes and induced a more rapid immune response than soluble GPIIb-IIIa. These antibodies reacted with GPIIb and IIIa by Western blot analysis. This immune response was anticipated in rabbits (in contrast to humans) since these are foreign proteins. Importantly, the antibody could be completely adsorbed by human platelets (figure 4). This suggests that neoantigens are not expressed by the purification of GPIIb-IIIa and insertion into a phosphatidylcholine bilayer.

PILA: clinical application Control thrombocytopenic sera evoked minimal glucose release (mean=0.15%, range 0-1.3% n=20). Sera from patients with ITP and platelet counts <40,000/ul or allo-immunization exhibited significant release: mean=6.7% and 8.1%, respectively (Table 11). The range in ITP was broad 0.7-9% which diminshed sensitivity; however, specificity was high. A release >5% was strongly indicative of circulating anti-platelet antibodies and >90% of patients with ITP had release >7%. Patients responding to therapy normalized release to control levels; relapse was associated with increased release.

DISCUSSION

In summary, this 2 year feasibilty study has demonstrated:

a. Feasibility of incorporation of GPIIb-IIIa into liposomes with retention of reactivity with monoclonal antibodies, and retention of FGN binding

b. Ability of GPIIb-IIIa incorporated onto the surface of liposomes to mediate FGN and Ca^{2+}, or cryoprecipitate dependent liposome aggregation

c. Ability to incorporate the fluorescent Ca^{2+} indicator, Fura-2, into liposomes and to utilize Fura-2-loaded-GPIIb-IIIa liposomes to demonstrate the ability of the GPIIb-IIIa complex to function as a Ca^{2+} channel which mediates the transit Ca^{2+} across a phosphatidylcholine bilayer

d. Inhibition of GPIIb-IIIa function as a Ca^{2+} channel by dissociation of the complex, by MoAb specific to the complex and by His-12-Val, a synthetic analogue of the gamma-COOH terminal of FGN and inhibition of the GPIIb-IIIa channel by La^{3+}

e. Ability of GPIIb-IIIa liposomes to adhere to deendothelialized bovine aorta in the presence of bound vWF and/or FGN and the inhibition of this adhesion by recptor blockade

f. Ability to induce thrombocytopenia in Sprague-Dawley rats with external Cesium irradiation while maintaining animal viability

g. Determination of the bleeding time in the Fawn-Hooded rat

h. Demonstration that these models can be used to screen the theraputic efficacy of liposome preparations

i. Identification of a liposome preparation that decreases bleeding in these animals but does not cause thrombosis in vivo as detremined by post mortem examination or in vitro assays

j. Developed an assay, the plateletsome immune lysis assay (PILA) based on this liposome model, to detect the presence of circulating anti-platelet antibodies

k. Determined that incorporation of GPIIb/IIIa into liposomes does not result in the expression of neo-antigens

l. Applied the PILA to common clinical syndromes

The complex nature of platelet physiology makes the development of an artificial platelet a formidable task. In this project, a determination of the minimum requirements for a platelet substitute, which would have some of the properties of a platelet,

and the feasibilty of a liposome based approach for this substitute are being evaluated. To this end, we have determined that platelet membrane glycoproteins can be successfully incorporated into an artificial membrane in a functionally active configuration. We have defined a Ca channel property for the platelet membrane GPIIb-IIIa complex in this model. While this observation is not directly related to the development of an artificial platelet, understanding the functional properties of GP after liposome incorporation is essential for such a transfusion modality.

A model for study of shear conditions has been developed. Adhesion of intact platelets to surfaces is among the most difficult of platelet functions to measure specifically. Conflicting experimental results are often obtained due to methodological differences rather than inherent platelet factors. Contributions from platelet release and aggregation, plasma factors, artifacts of platelet preparation, anticoagulants, and pH are seen with in vitro platelet studies. Use of liposomes with incorporated GP precludes many of these problems. In this model, GPIIb-IIIa-liposomes can adhere to subendothelium under non-shear conditions. The data indicate that, with shear, GPIIb-IIIa liposome adhesion is less than under non-shear conditions. Adhesion is one of the minimum requirements for a platelet substitute. In addition, GPIIb-IIIa on a liposome surface can bind appropriate ligand, another essential requirement for a platelet substitute. However, our data indicate that plasma inhibits both of these activities. This is apparently related to receptor blockade due to the circulating ligands (vWF and FGN) found in plasma at levels significantly above their respective Kd's. Thus, as anticipated, additional surface constituents are necessary to facilitate adhesion at a wound surface in vivo. GPIb is a promising candidate since this GP does not bind soluble ligand. We have developed a procedure for purification of active GPIb in sufficient, albeit still limited, quantities for evaluation in liposomes. However, the need for fresh human platelets and relatively low yield of procedures to prepare this GP makes it relatively impractical for a platelet substitute. Because of cost limitations, GPIb was not further pursued in this 2 year study. When recombinant GPIb is a practical alternative, such protein may be important in an artificial platelet. For these reasons, a plateletsome prepared from several platelet proteins which can be prepared from out-dated human platelets is an attractive option. In the current contract period we have shown that a composite plateletsome preparation can decrease blood loss in animal models of thrombocytopenia and thrombocytopathy.

Participation in platelet aggregate fromation without directly activating platelets is an important criteria for a platelet substitute. The composite and GPIIb-IIIa plateletsomes have that property. When subjected to centrifugation, in the presence of appropriate ligands, these plateletsomes will form aggregates. This demonstrates the availability of appropiate receptors on the plateletsome surface. The need for centrifugation and the ease of

disruption by shear forces indicate that other surface constituents would be necessary for stable aggregates in vivo. The same observations suggest that significant spontaneous aggregates, which could have deleterious in vivo consequences, do not form.

At the present time, an important consideration in the development of a liposome based platelet substitute is the potential expression of new antigens by the purification and liposome insertion of glycoproteins. The data from rabbits immunized with soluble GPIIb-IIIa, liposomal GPIIb-IIIa, and human platelets suggest that new antigens are not expressed as a consequence of insertion of these GP into an artificial phosphlipid membrane. Data suggest that the current composite plateletsome is not associated with neoantigens. The current study was limited to the development of complement fixing antibodies which would be of clinical significance; however, more extensive study is required. When a final effective platelet substitute is developed, the ultimate determination of antigenicity must be made in the clinical situation, analogous to the approach used with the administration of monoclonal antibodies and recombinant proteins to patients. Nevertheless, the current data are promising. In addition, the PILA developed for this study will detect over 90% of patients with ITP and is very sensitive in detecting alloimmunization which are two important clinical problems. A modification assay could be ultimately be used to screen potential platelet and platelet substitute recipients for antibody.

Proteoliposome stability in plasma is another important consideration. The reverse-phase methodology generates proteo-liposomes which are stable in plasma for up to 14 days. This is adequate for clinical administration; liposomes rapidly adhere to a wound site or be cleared by the reticulo-endothelial system before inherent stability problems emerge. The stability observed with this preparation both in plasma and in buffer is inadequate for long term storage of an effective moiety. Thus preservation methods such as lyophilization will be necessary. This was addressed in our initial proposal but at the suggestion of the reviewers this objective was not pursued in this contract period.

Another consideration for a platelet substitute is the possiblity of massive activation of coagulation following in vivo administration. In vitro and in vivo evaluation of proteo-liposome preparations to this point have shown no evidence of such activation.

In order to assess the efficacy of a platelet substitute, an in vivo model is essential. Such a model requires reproducibility, independence from humoral coagulation, thrombocytopenia or severe platelet dysfunction, and a method of evaluation of primary (platelet) hemostasis. The rat tail bleeding time, under carefully controlled conditons, in the irradiated thromboctytopenic rat and the thrombocytopathic rat are such models. We have demonstrated the ability to induce consistent, prolonged thrombocytopenia in rats with Cesium irradiation which preserves humoral coagulation,

in contrast to exchange transfusion which requires plasma administration to compensate for coagulation factor depletion, and to other models of thrombocytopenia e.g which require the administration of anti-platelet antibodies which would also effect plateletsomes. The tail vein bleeding time in these animals which is consistently prolonged (>22 times control) and which is associated with a marked increase in blood loss (>20 times control), can provide such an index of efficacy of a platelet substitute. This model will provide a primary screen for efficacy; however, a model representative of the trauma situation with multiple sights of bleeding, will be required once an efficacious substitute in the current model is developed.

Despite formidable theoretical obstacles, we were able to define a plateletsome preparation which significantly shorten the bleeding time in both thrombocytopenic and thrombocytopathic animals. This plateletsome preparation in the doses studied did not completely normalize the bleeding in these animals; however, the 42% decrease in total blood loss is extremely encouraging and strongly supports the feasibility of this approach.

Conclusions and Future Directions

In summary, the goals of this 2 year contract a defined in the Army Research and Development Command letter of May 22, 1987, to conduct a feasibility study which would demonstrate that platelet membrane proteins could be incorporated into a lipid bilayer with functional intergrity and out-side out orientation, that would address lipid effects on function, that would address antigenicity due to neo-antigen expression, that would address stability and the potential for massive activation of coagulaton have been accomplished. In addition, an important property, i.e. calcium channel function, of the platelet membrane GPIIb-IIIa complex has been described. Animal models for the study of future platelet substitutes have been developed and characterized, and a new, sensitive assay for the presence of anti-platelet antibodies has been developed. In addition, the critical observation that a liposome based modality can shorten the bleeding time in the model system has been made.

Future studies can be directed at enhancement of the efficacy of this liposome-based modatily. This can be done with further studies on the function of platelet GP in liposomes, improved preparative methods for both GP and liposomes to enhance these functional properties, definition of the biodistribution of effective plateletsomes and methodology to enhance stability and survival in vivo, e.g use of sphingomyelin and gangliosides, exploration of surface contituents to enhance function, e.g. the platelet collagen receptor which has recently been purified and incorporated into liposomes, and non-platelet derived components, for example, monoclonal antibodies to subendothelial constituents. In addition, platelet glycoproteins produced by recombinant technology which could potentially overcome several of the

limitations of purification of proteins from platelets, will need to be evaluated functionally in liposomes.

Thus, while significant obstacles exist, a number of potentially fruitful avenues are emerging for the ultimate goal of developing a platelet substitute.

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Abbreviations Used

AA:	Amino Acid
Ab:	Antibody
ADP:	Adenosine diphosphate
BSA:	Bovine serum albumin
BT:	Bleeding time
$[Ca^{2+}]_i$:	Intracellular ionized Ca^{2+} concentration
CF:	Carboxyfluorescein
CHO:	Carbohydrate
CRYO:	Cryoprecipitate
DOC:	Deoxycholate
EGTA:	Ethylene-bis(oxethylenenitrilo)tetraacetic acid
ELISA:	Enzyme linked immunoassay
F-Ab:	Fluorescein conjugated Ab
FGN:	Fibrinogen
F-H:	Fawn-Hooded rats
FNC:	Fibronectin
GFP:	Gel filtered platelets
GP:	Glycoprotein
GC:	Glycocalicin
Hgb:	Hemoglobin
His-12-Val:	Dodecapeptide His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Val
MoAb:	Monoclonal antibody
NEM:	N-ethylmaleimide
PC:	Phosphatidylcholine
PBS:	Phosphate buffered saline
PILA:	Plateletsome immune lysis assay
PS:	Phosphatidylserine
RT-BT:	Rat tail bleeding time
RGDS:	Tetrapeptide Arg-Gly-Asp-Ser
SDS:PAGE	Sodium dodecylsulfate polyacrilamide gel electrophoresis
TPA:	Human tissue plasminogen activator
TSE:	10mM Tris buffer, pH 7.4, 140mM NaCl, 1mM EDTA

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Articles

1. Rybak ME, Renzulli L: Ligand Inhibition of the Platelet Glycoprotein IIb-IIIa Complex Function as a Calcium Channel in Liposomes. Jour Biol Chem 264:14617-14620, 1989.

2. Rybak ME, Baca M, Renzulli L: Independence of Platelet Membrane Glycoprotein IIb and IIIa antigenicity from Lipid Milieu (in preparation).

3. Rybak ME, Linden H, Baca M, Renzulli L: A Liposome-based Assay to Detect Immune Mediated Thrombocytopenia (In preparation).

4. Rybak ME, Renzulli L, Baca M: The thrombocytopenic rat: A model of abnormal hemostasis. (In preparation).

Abstracts

1. Rybak ME, Renzulli L: Differential effect of ligand binding on platelet glycoprotein IIb-IIIa function as a Ca^{2+} channel. Circ 78, Supp 2, 1988.

2. Rybak ME, Linden H, Baca M: Diagnostic assay for thrombocytopenia: the plateletsome immune lysis assay (PILA). NE Canc Soc (in press).

Table 1

Outline of revised proposal

Section 1 Theoretical Considerations and Experimental Plan

fI. Preparation of liposomes which bind to components of subendothelium exposed at a wound surface.
- A. Proteins to be assessed
  - a. Glycoproteins IIb and IIIa
  - b. Glycoprotein I complex
  - c. Combination of a and b
- B. Stability of proteins in membranes
- C. Protein-protein interactions

II. Effects of lipid composition on protein function

III. Effects of protein orientation on protein function

IV. Immunogenicity

V. Massive activation of coagulation in vivo

Section 2 Methods

I. Glycoprotein purification
- A. GPIIb-IIIa
- B. GPI

II. Plateletsome preparation
- A. Primary method
- B. French press modification
- C. Lipid composition
- D. Stability

III. Activation of coagulation by plateletsomes
- A. Effect on humoral coagulation
  1. in vitro
  2. in vivo
- B. Effect on platelet aggregation
  1. in vitro
  2. in vivo

IV. Immune lysis assay

V. Adhesion Studies
- A. Microtiter wells
- B. Immobilized collagen
- C. Denuded aorta

VI. In vivo hemostasis studies
- A. Rat
- B. Rabbit

Table 2

Binding of MoAbs to GPIIb-IIIa liposomes by total FITC-fluorescence (ex 495nm, em 525nm) of liposomes after incubation with specific monoclonal antibodies and irrelevant antibody plus FITC coupled anti-murine antibody or FITC anti-murine antibody alone.

Antibody	F	Source
M148	35.8 ± 5.1	Hardisty
7E3	27.6 ± 4.0	Coller
10E5	19.4 ± 3.1	Coller
AP2	21.4 ± 2.1	Kunicki
357 (irrelevant antibody)	13.2 ± 1.0	Herrman
FITC antimurine	15.5 ± 1.2	

Specific antibody fluorescence compared to irrelevant antibody or FITC-anti murine antibody was significant to $p<0.005$. Specific antibody exhibited only background activity when incubated with control (PC only) liposomes.

Table 3
Aggregation by liposome type and addition

Liposome type	Addition	Aggregates
control liposomes	Cryoprecipitate	1+
	FGN + FNC + VWF	1+
	FNC	1+
	vWF	1+
IIb-IIIa liposomes	Cryoprecipitate	3+
	FGN + FNC + vWF	3+
	FGN + FNC	3+
	FGN	2-3+
	vWF	1+
	FNC	1+
IIb-IIIa liposomes+ Monoclonal antibody (10E5 or 7E3)	cryo	1-2+

Legend: FGN: Fibrinogen, FNC: Fibronectin, vWF: von Willebrand factor.

Table 4: Change in Intravesicular Fluorescence (ΔF) and Intravesicular Calcium ($[Ca^{2+}]_i$) Due to Ca^{2+} Movement Into IIb-IIIa Complex, Dissociated IIb/IIIa, and Nonprotein Liposomes

External $[Ca^{2+}]$ mol/L	IIb-IIIa		Dissociated IIb/IIIa		Lipid Vesicles (ΔF)
	ΔF	$[Ca^{2+}]_i$ (mol/L)	ΔF	$[Ca^{2+}]_i$ (mol/L)	
10^{-6}	4.1 ± 2.0	0.7×10^{-6}	0.5 ± 0.1	0.04×10^{-6}	0.5 ± 0.1
10^{-4}	8.2 ± 3.1	0.6×10^{-4}	0.15 ± 0.2	0.06×10^{-6}	0.7 ± 0.2
10^{-3}	18.5 ± 4.2	2.0×10^{-3}	1.0 ± 0.5	0.10×10^{-6}	1.0 ± 0.2
10^{-2}	19.6 ± 2.0	4.0×10^{-3}	1.2 ± 0.5	0.11×10^{-6}	1.0 ± 0.3
10^{-2} + ionomycin (1 μmol/L)	23.4 ± 2.9	1×10^{-2}	23.4 ± 2.1	1×10^{-2}	22.4 ± 2.1

Difference between complexed and dissociated proteins, $P < .0001$; difference between 10^{-6} and 10^{-4}, $P < .05$; difference between 10^{-4} and 10^{-3}, $P < .0001$; difference between 10^{-3} and 10^{-2}, not significant.

Table 5
Liposome stability by carboxyfluorescein release

MEDIA:	HEPES/NaCl pH 7.4		PLASMA	
Liposome Compostion:	100% PC	PC:Chol	100% PC	PC:Chol
Day	% Release			
0	0	0	0	0
2	0	0	20	0
7	8.4	12	24.5	0
10	18	23	12.5	0
14	18	-	17.8	5.8
21	36.8	37	20	12
30	50	15	37	60
42	Not done liposome suspension totally fused.			

Legend

PC = Phosphatidylcholine

CHOL = Cholesterol

PC: CHOL = 30mole% PC:30mole% cholesterol

Table 6
Liposome Size-nm over time with plasma or buffer incubation

	Buffer		Plasma	
	PC	PC:CHOL	PC	PC:CHOL
Day				
0	4250	2620	2960	2060
2	4400	4920	2960	2060
7	12900	1120	5850	3051
10	18800/3540*	2750	2400	3510
14	14100	10800	17900/6060*	9130
21	9270/3920*	5190	18500	28100/ 7590*

Day 30-42: results invalid since liposomes totally fused.

* 2 populations of liposomes present

Table 7

Liposomes were added to plasma or whole blood and prothrombin time (PT), partial thromboplastin time (PTT) or whole blood clotting time (WBCT) performed by standard methods.

Plasma (ul)	liposomes (ul)	PT (sec)	PTT(sec)
100	0	12.0	24.6
100	20	12.0	23.0
100	40	12.4	23.8
100	80		23.8
100	100		25.9

Whole blood clotting time

Whole blood		WBCT
500ul	0	135±10sec
500ul	20	120±12sec

n=10
no significant differences were seen among the values.
S.D pf PT and PTT±0.5 sec

Table 8

Adhesion of ^{3}H-liposomes under non-shear conditions to deendothelialized aorta with and without cryoprecipitate. A suspension of liposomes ($100x10^{3}$ cpm, 12ug lipid) was incubated with the substrate indicated. Wells were washed and bound lipid determined.(detailed in Methods)

Liposome	Control %Adhesion	Control (ng lipid)	IIb-IIIa %Adhesion	IIb-IIIa (ng lipid)
Aortic Segments				
Bovine	0.816	(84)	1.6	(505)
+ cryo	2.1	(505)	6.9	(1663)
	p=nsd		p<0.001	
Human				
+ cryo	3.69	(885)	5.35	(1283)
			p<0.09	
Preincubation of liposomes with plasma human aorta+cryo			1.4	(485)
			p<0.001 compared with cryo+no preincubation	
Microtiter wells				
BSA		(34)		(34.9)
FGN		(32)		(42.5*)
FNC				
75 ug/ml		(30)		(30.7)
250 ug/ml		(30)		(24.9)
Collagen				
Type IV				(28.2)
Calf Skin				(28.6)

* p<0.005 compared to BSA or collagen

Table 9
Thrombocytopenia

Induction of thrombocytopenia is rats by external total body irradiation with a Cesium source: platelet count as a function of radiation dose.

RADIATION DOSE	200	400	600	900	Control
			platelet count/ulx10^{-3}		
Day					
4	658	484	547	407	873
10	161	227	67	19	1,184
11	486	299	107	18	1,167
12	604	250	190	19	1,044
14	984	506	548	17	1,128
17	1299	972	1293	-	1,320

Table 10

Rat tail bleeding times in control, thrombocytopenic and thrombocytopathic rats

RT-BT were performed in sets of animals before and after infusion of : 2ml buffer, control (PC) liposomes or composite liposomes as detailed in Experimental Methods.

	BT	Blood Loss (ul) 30 sec	60 sec	6 min
Normal rats**	113 ± 3 sec	19	-	13
Thrombocytopenic* rats				
control	>6 min	102.9 ± 30.3	223.8 ± 58.5	369.7±59.8
Infusion PC-liposomes/buffer	>6 min	102.5 ± 29.5	223.4 ± 58.2	369 ± 59.8
Composite liposomes	>6 min	47.6 ± 21.8 p<0.01	73.8 ± 22.4 p<0.005	212 ± 54.3
Fawn-Hooded control	>6 min	143.2 ± 20	212 ± 30ul	458.3 ± 60
Infusion buffer control**	>6 min	167.2 ± 40	204 ± 31ul	460.4 ± 55
Composite liposomes	>6 min	91.3 ± 10 p<0.005	140.2 ± 20 p<0.05	280 ± 80

* platelet count 20-30,000/ul
** buffer infusion had no significant impact on BT or blood loss
BT: bleeding time
PC-Liposomes: phosphatidylcholine liposomes
RT-BT: rat tail bleeding time
p values -comparison with control

Table 11

PILA STUDY

Serum samples were obtained from patients with thrombocytopenia. Glucose release by serum antibody was measured as detailed in Methods.

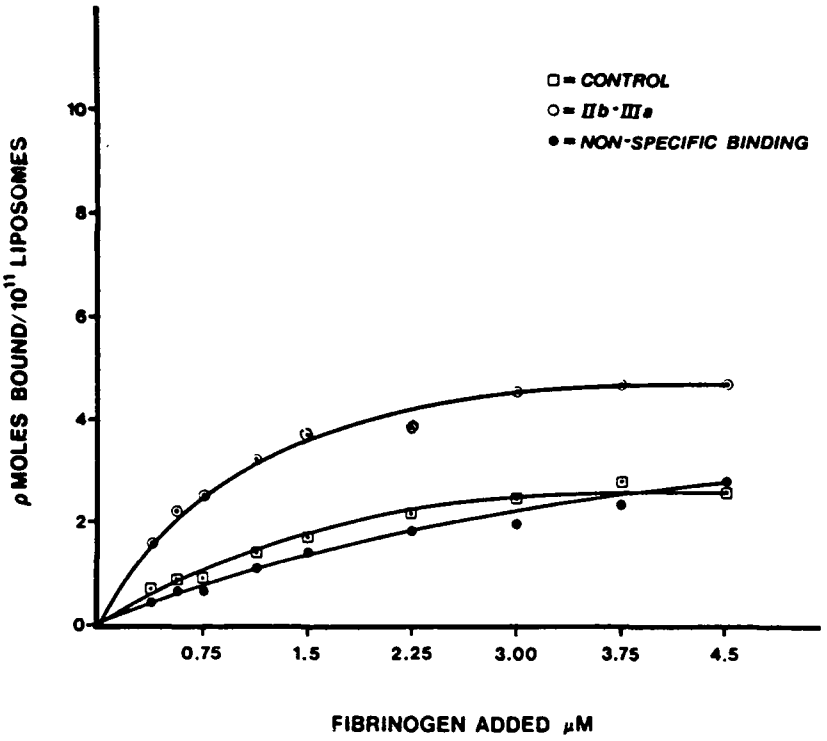
	n	Platelet Count	% Glucose Release	
		$x10^3/ul$	30'	1h
Controls*	11	19 - 27	0.15 ± 0.39*	0.25 ± 0.58*
ITP	8	6 - 97	6.21 ± 3.7	5.13 ± 3.6
Alloimmunized	6	8 - 72	8.2 ± 4.8	7.31 ± 6.3

*Controls: non-immune thrombocytopenia

** $p<0.001$ compared to ITP or compared to alloimmunized

ITP: immune thrombocytopenia
PILA: plateletsome immune lysis assay

Figure 1



^{125}I-fibrinogen binding to IIb-IIIa liposomes. 250 ul of liposomes (1×10^5 liposomes/ul) of control liposomes or IIb-IIIa liposomes were incubated with ^{125}I-fibrinogen for 30 min at 37° then washed in 4% sucrose with centrifugation at 10,000 xg. Non-specific binding was defined as binding in the presence of 10 mM cold fibrinogen (●—●). Specific IIb-IIIa liposomes fibrinogen binding (○—○) is the net difference of total and non-specific binding. Control liposomes manifest only non-specific binding (□—□)

Figure 2

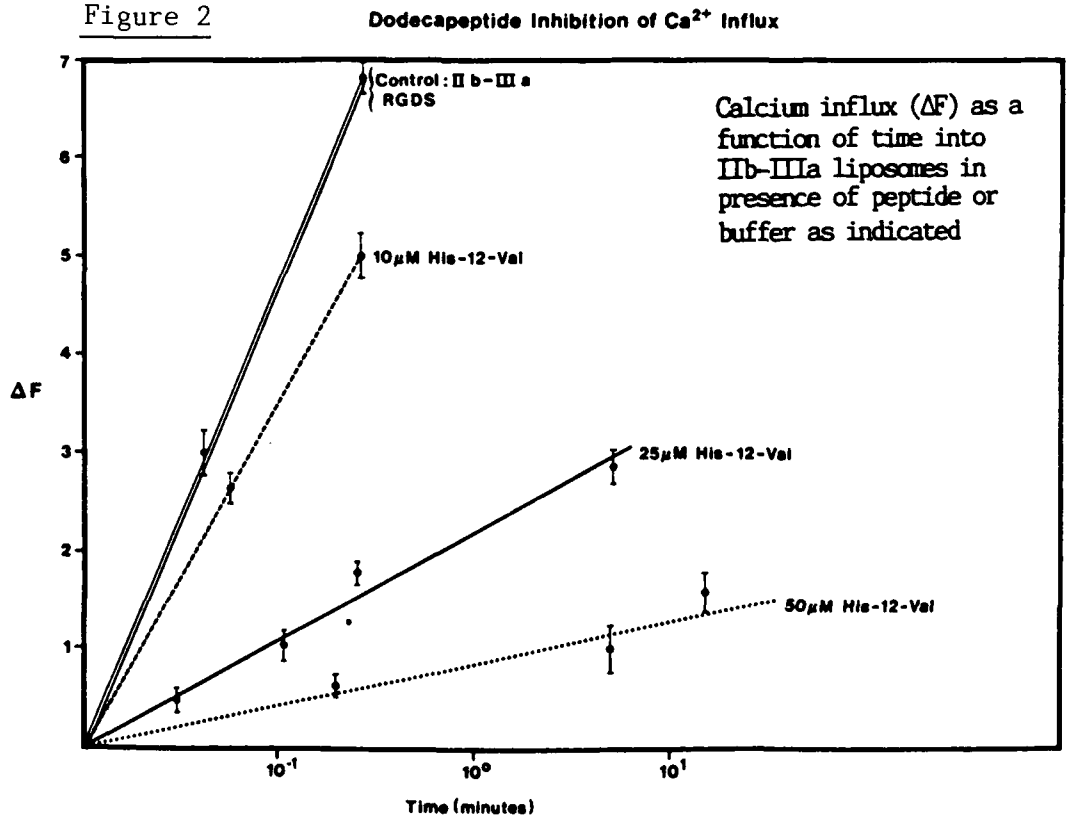
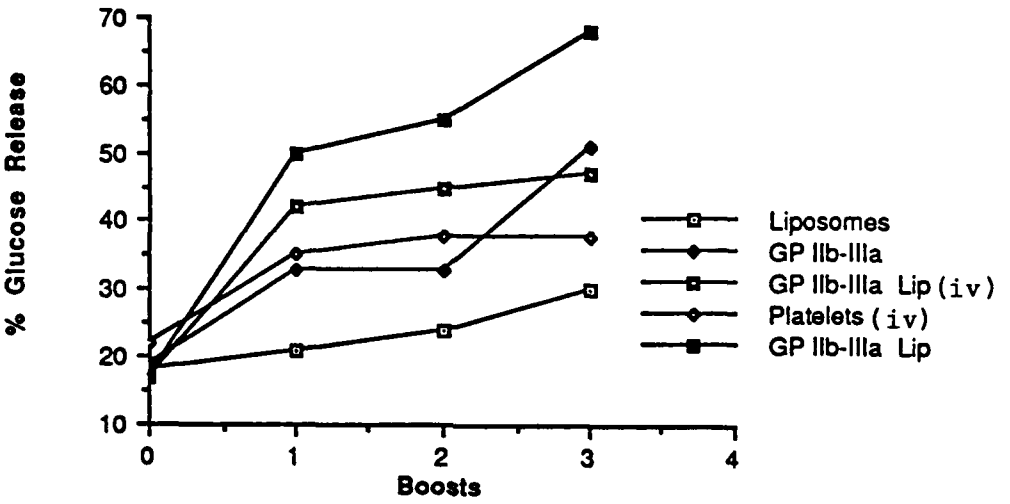


Figure 3

Release of Glucose from Liposomes by Rabbit Sera from Animals Immunized with Different Immunogens

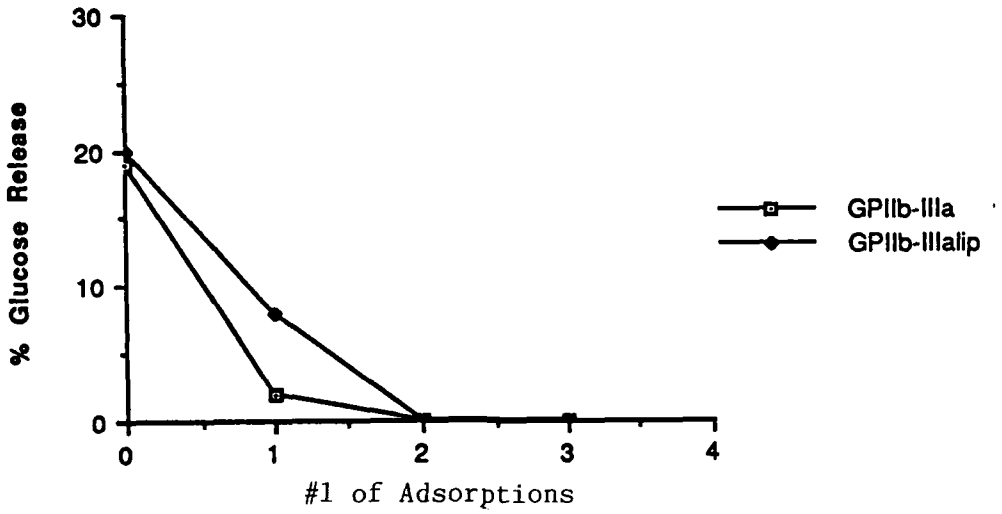


Immune Lysis Assay was performed on rabbit sera adsorbed with human RBC, at the time points indicated, after immunization with the immunogen noted.

GPIIb-IIIa Lip:	GPIIb-IIIa in liposomes
GPIIb-IIIa:	Soluble GPIIb and IIIa

Figure 4

Immune Lysis Assay Activity after
Adsorption with Human Platelets



Residual activity in Immune Lysis Assay after adsorption with human platelets, for sera from rabbits immunized with soluble GPIIb-IIIa or with GPIIb-IIIa liposomes.